Epitope Location in the *Cryptococcus neoformans* Capsule Is a Determinant of Antibody Efficacy

By Gabriel Nussbaum,* Wendy Cleare,‡ Arturo Casadevall,‡ Matthew D. Scharff,* and Philippe Valadon*

*From the *Department of Cell Biology and the ‡Division of Infectious Diseases, The Department of Medicine, the Albert Einstein College of Medicine, Bronx, New York 10461*

Summary

Monoclonal antibodies (mAbs) to the polysaccharide capsule of *Cryptococcus neoformans* can prolong survival in mice. However, the properties of antibodies that mediate protection are not fully understood. The IgM mAbs 12A1 and 13F1 originated from the same B cell and differ only by somatic mutations in their variable regions; yet mAb 12A1 protects against serotype D infection, while mAb 13F1 does not. Phage peptide display libraries were used to analyze the fine specificity of these two mAbs. The selection of distinct peptide motifs from identical libraries confirmed that mAbs 12A1 and 13F1 bound to two distinct epitopes. Immunofluorescence and immunoelectron microscopy studies revealed differences in antibody localization within the capsule of serotype D strain; mAb 12A1 bound to the outer rim of the capsule resulting in an annular pattern, whereas mAb 13F1 bound throughout the capsule and had a punctate appearance. The difference in the binding pattern of mAb 12A1 and 13F1 was not observed on serotype A organisms, where both mAbs bound to the capsule with an annular fluorescence pattern. The fluorescence pattern of binding correlated with protective efficacy; mAb 13F1 prolonged survival of mice infected with the J11 serotype A strain (annular fluorescence), but not serotype D strains (punctate pattern). Annular binding, but not punctate binding, was associated with increased opsonic efficacy for phagocytosis of *C. neoformans* by J774.16 macrophage-like cells. The correlation between capsular binding pattern, opsonic activity, and ability to prolong survival suggests that the efficacy of anticryptococcal antibodies is dependent upon where they bind in the polysaccharide capsule.

The concept of protective and nonprotective epitopes emerged from studies on the interaction of viruses with particular antibodies (1). The efficacy of mAbs in modulating bacterial infections can depend on the epitope that the mAb binds to the bacterial surface (2). While the value of antibodies in the host defense against bacteria and viruses is accepted, the role of antibodies against medically important fungi remains controversial (3). Much of the initial evidence supporting or contradicting a role for antibodies in the defense against fungi relied on experiments using polyclonal sera which contained complex mixtures of antibodies differing in epitope specificity and isotype, both of which may determine antibody efficacy (3). More recently, using mAbs, protective, and nonprotective antibodies to *Candida albicans* and *Cryptococcus neoformans* have been identified (4, 5).

C. neoformans frequently causes a fatal meningoencephalitis in patients with AIDS. In New York City alone, there were over 1,200 cases in 1991, with a prevalence of infection in patients with AIDS is 6–8% (6). Many cases are incurable because antifungal therapy fails to eradicate infection in the setting of severe immunosuppression. *C. neoformans* is unusual among fungi in that it has a polysaccharide capsule. The polysaccharide capsule blocks phagocytosis (7) and the capsular polysaccharide is shed into the circulation and tissues during infection. Soluble polysaccharide may contribute to virulence by suppressing the immune response (8), inhibiting leukocyte migration (9), and enhancing HIV infection (10). mAbs that bind the polysaccharide capsule can enhance in vitro phagocytosis (11), reduce serum polysaccharide (5), and prolong in vivo survival in murine infection models (12). We have previously demonstrated that antibody isotype and epitope specificity are important determinants of antibody protective efficacy. For example, murine IgG3 antibodies enhance infection and block IgG1- and IgG2a-mediated protection (13, 14).

A role for epitope specificity in determining protective efficacy was suggested by experiments with two murine IgM anticryptococcal mAbs, 12A1 and 13F1. These mAbs originated from the same B cell but differed in their reactivity with cryptococcal polysaccharide and their ability to prolong the survival of mice lethally infected with a serotype D strain (5). mAbs 12A1 and 13F1 were generated in

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response to immunization with glucuronoxylomannan (GXM)[1], the primary component of the cryptococcal polysaccharide capsule, conjugated to tetanus toxoid (GXM-TT). Their V_H regions differ by five amino acids in the first and second CDRs and three amino acids in framework regions, and their V_L regions differ by one amino acid in CDR1, one amino acid in CDR2, and three amino acids in framework regions (15). Indirect immunofluorescence revealed differences in binding to the polysaccharide capsule by mAbs 12A1 and 13F1 (5). The protective mAb, 12A1, produced a homogeneous annular fluorescence pattern, whereas the nonprotective mAb, 13F1, produced a punctate pattern of fluorescence on one strain of serotype D, *C. neoformans.*

Because these preliminary observations seemed to indicate structural heterogeneity in the capsule of serotype D organisms that was associated with the protective efficacy of antibodies, we studied other strains of *C. neoformans* representing all serotypes. We report here that the antibody binding pattern differed among the four *C. neoformans* serotypes and that the location of IgM binding appears to be critical for protective efficacy, suggesting a relationship between the ability to confer protection and the location of antibody binding to the cryptococcal capsule. Since oligosaccharides are not available to define the chemical structure of the protective and nonprotective polysaccharide epitopes, we screened phage peptide display libraries with mAbs 12A1 and 13F1 and defined peptide binding motifs that distinguished the binding sites of the two antibodies. Selected peptides were used to probe the antibody-binding pocket and confirmed differences in the fine specificity of mAbs 12A1 and 13F1.

Materials and Methods

C. neoformans. Strains 24064, 24065, 24067, 32608, 34873, and 34874 were obtained from the American Type Culture Collection (Rockville, MD). Strains J11, J22, and SB6 were isolated from the cerebrospinal fluid of patients with cryptococcal meningitis and have been described (16). Strain National Institutes of Health 371 was obtained from Dr. J.E. Bennett (Bethesda, MD). All strains were maintained on Sabouraud dextrose agar slants (Difco Labs., Detroit, MI) and grown in Sabouraud dextrose broth (Difco Labs.) at 37°C before use. (For strain serotypes see Table 2.)

mAbs. mAbs 12A1, 13F1, and 21D2 were described previously (17, 18). Ascites was generated by injecting 10^7 hybridoma cells into the peritoneal cavity of pristane primed BALB/c mice. Antibody concentration was determined by ELISA relative to isotype-matched standards of known concentration. Lipid was removed from ascites samples (Cleanascite; Affinity Technology Inc., New Brunswick, NJ) and ascites was used directly in protection experiments. For in vitro phagocytosis and phage ELISA studies, IgMs were purified by mannose-binding protein affinity chromatography (Pierce Chem. Co., Rockford, IL).

Selection of 12A1 and 13F1 Binding Phage. Antibody binding phage were isolated from random hexapeptide and decapeptide libraries by multiple cycles of selection and amplification (19). The hexapeptide library was reamplified from an aliquot provided by Dr. George Smith (University of Missouri, Columbia, MO). We constructed the decapeptide library with the filamentous phage fUSE5 vector (20). In the initial screening, mAbs 12A1 and 13F1 were attached to streptavidin-coated magnetic beads (Adv. Magnetics, Inc., Cambridge, MA) with a biotinylated anti–mouse IgM antibody (Southern Biotechnology Assoc., Birmingham, AL). All incubations were at room temperature in biopanning buffer (BPB) (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (wt/vol) BSA, 0.1% (vol/vol) Tween 20, and 0.02% (wt/vol) NaN_3) with slow end-over-end rotation. 0.2 μM IgM was first incubated for 30 min with 1.5 μg of biotinylated anti–mouse IgM antibody in 50 μl BPB. The mixture was transferred to a microfuge tube containing 50 μl of streptavidin-coated beads (storage solution was removed with a magnet from Dynal, Oslo, Norway). After 30 min, the beads were vortexed and the remaining biotin binding sites were blocked with 1.5 μl of D-biotin (10 mM) for 10 min. The beads were washed three times with 500 μl BPB, and then incubated for 4 h with 200 equivalents of peptide library (4×10^{10} transducing units [TU] for the hexapeptide library and 8×10^{10} TU for the decapeptide library [sublibraries L102 and L103]; reference 20). The beads were washed seven times with BPB during which they were transferred three times to new siliconized microfuge tubes. Bound phage particules were eluted at pH 2.2 as described (21). Phage titration, amplification, and polyethylene glycol purification were done according to Smith and Scott (22). For subsequent rounds of selection, $\sim 1 \times 10^9$ TU from the preceding round were first incubated for 4 h with lower concentrations of IgM mAb, and then phage antibody complexes were captured for 10 min by the addition of 10 μl streptavidin-coated magnetic beads saturated with 3 μg biotinylated anti–mouse IgM antibody.

Sequence Determination of Displayed Peptides. Phage DNA was purified by extraction of the virion preparation with phenol and chloroform, and re-suspended in 7 μl of water after ethanol precipitation. Dideoxy sequencing reactions (23) were performed using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) with a ^{32}P end-labeled 18-base fUSE5 primer (22). Termination reactions were started directly in microwells of microtesting plates (Robbins Scientific Corp., Sunnyvale, CA). Samples were run on polyacrylamide gels (6% monomer) using Sequagel sequencing reagents (National Diagnostics, Inc., Atlanta, GA).

Phage ELISA. Antibody reactivity with phage was detected by the "direct" ELISA as described (24). In brief, phage were prepared from overnight cultures by two polyethylene glycol precipitations according to published methods (22). Phage were captured on the ELISA plate using an anti-m13 antibody (5 Prime–3 Prime, Inc., Boulder, CO). Phage (5×10^{10} virions) were incubated in the ELISA well for 2 h, and after washing, mAb (0.5 μg/ml) was incubated with phage for 2 h. After washing, anti–mouse IgM antibody conjugated to alkaline phosphatase was added and incubated for 1 h. Phosphatase substrate *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) was dissolved in diethanolamine buffer, pH 9.8, to a concentration of 1 mg/ml and 50 μl/well were added to the plates. Absorbance at 405 nm was measured in a microplate reader (EL 320; Biotek Instrument Inc., Winooski, VT) after 1 h of incubation at 37°C. Each test was done in duplicate, and the absorbance given by the control phage was subtracted. Phage ϕ673 is a randomly selected recombinant phage bearing the sequence RKVWVI which was used as a control in the hexapeptide phage ELISAs. Phage ϕ33 does not contain an insert and was used as a control phage in the decapeptide phage ELISAs.

[1] *Abbreviations used in this paper:* BPB, biopanning buffer; GXM, glucuronoxylomannan; GXM-TT, GXM conjugated to tetanus toxoid; IEM, immunoelectron microscopy; TU, transducing units.

Indirect Immunofluorescence. Stationary phase organisms were washed three times in PBS, and 1×10^6 organisms were placed into microfuge tubes. mAb was added to the organisms (5 μg/ml) and incubated at room temperature for 1.5 h (identical results were obtained at 4°C). An IgM standard (Organon Teknika, Durham, NC) was used as a negative control. Organisms were collected by centrifugation, washed twice in PBS/1% (wt/vol) BSA, and resuspended in FITC-labeled goat anti–mouse IgM antibody (Southern Biotechnology Assoc.) for 1 h at room temperature in the dark. Organisms were washed twice in PBS/BSA and resuspended in 50 ml mounting media (50% [vol/vol] glycerol/PBS, 0.1 M *n*-propyl gallate) (Sigma Chemical Co.). The suspension was mounted on poly-l-lysine–coated slides (Sigma Chemical Co.) and viewed with a Zeiss phase contrast microscope equipped with a FITC filter or with a confocal microscope (MRC 600; BioRad Labs., Hercules, CA) using Nikon ×60 numerical aperture 1.4 planapochromat optics and a Kr/Ar laser. For confocal microscopy, 16 optical sections were collected at 0.8 μm intervals and imaged by the maximum projection method. Confocal microscopy was carried out (Analytical Imaging Facility of the Cancer Center of the Albert Einstein College of Medicine, New York).

Immunoelectron Microscopy. Cryptococci were prepared for immunoelectron microscopy (IEM) in the same manner as for immunofluorescence. Goat anti–mouse IgM antibody coupled to 10 nm gold balls (E-Y Laboratories, Inc., San Mateo, CA) was diluted 1:30 in PBS/BSA and centrifuged for 2 min after which the supernatant was used. Organisms coated with IgM mAbs 12A1, 13F1, and an irrelevant IgM used as a negative control were resuspended in 100 μl gold labeled antibody for 1 h at room temperature. Labeled organisms were washed and resuspended in electron microscopy fixative (Trumps solution composed of 1% [vol/vol] glutaraldehyde and 4% [vol/vol] paraformaldehyde in PBS), embedded in plastic, cut into sections, and examined in the Analytical Imaging Facility of the Cancer Center of the Albert Einstein College of Medicine.

Animal Studies. All protection experiments were done using female A/J mice aged 6–8 wk (Jackson Labs., Bar Harbor, ME). mAb (1 mg per mouse) was administered intraperitoneally ~30 min before infection. *C. neoformans* infection was induced by intraperitoneal inoculation using an inoculum of 1×10^8 yeast cells for the serotype D experiment and 3×10^7 yeast cells for the serotype A experiment. Deaths were recorded daily and data was analyzed by log-rank analysis (Instat version 2.01 for Macintosh; GraphPAD Software for Sci., San Diego, CA).

Phagocytosis Assay. The ability of mAbs 12A1 and 13F1 to promote phagocytosis of *C. neoformans* strains 24067 and J11 was studied using the J774.16 cells as described (11, 25). In brief, J774.16 cells were plated on tissue culture plates (No. 3695; Costar Corp., Cambridge, MA) and stimulated with 500 U of IFN-γ (Genzyme Corp., Cambridge, MA) and 3 μg/ml of LPS (Sigma Chemical Co.). *C. neoformans* was washed and resuspended in sterile PBS before adding to the J774.16 monolayers in a macrophage to yeast ratio of 1:1, with either mAb 12A1 or 13F1. The suspension was incubated for 2 h at 37°C. The J774.16 monolayers were then washed several times with PBS to remove nonadherent organisms, fixed with cold absolute methanol, and stained with a 1:20 solution of Giemsa (Sigma Chemical Co.). The phagocytic index is the number of internalized yeast per number of macrophages per field. For each mAb, five to six fields were counted, each containing at least 200 cells. Previous studies with diaethanol staining and electron microscopy have established that light microscopy can distinguish between internalized and attached yeast cells (11). Phagocytic indices were determined with a microscope at the magnification of ×600 (Nikon Diaphot; Nikon Inc., Instr. Group, Melville, NY).

Results

Peptide Mimotopes Selected by mAbs 12A1 and 13F1. While the results presented in previous studies (5) strongly suggested that mAbs 12A1 and 13F1 bound to different epitopes, differences in their fine specificity could not be confirmed using oligosaccharides since none have been identified that react with antibodies to the capsular polysaccharide. As an alternative, we screened phage libraries containing either 6 or 10 random amino acid inserts in the pIII coat protein of filamentous phage fd (19). After three rounds of selection from the hexapeptide library with 0.2 μM IgM, mAb 12A1 selected two phage with related peptide sequences (ϕM1 and ϕM2) which differ at 2 positions (Table 1). mAb 13F1 selected two unrelated peptide sequences that did not bind 13F1 in the phage ELISA (sequences not shown). The low ELISA signals and the lack of sequence diversity in the selected peptides suggested that a larger peptide was required to find higher binding motifs, a conclusion that we had already come to with the related mAb 2H1 (20).

We therefore screened a decapeptide phage library. After a first round with each mAb at 0.2 μM and a second at 0.2 nM, the phage yield for 12A1 was 1.2% in the presence of antibody compared to 0.06% without antibody, and for 13F1, 33% compared to 0.03% without antibody. Randomly selected phage were sequenced at this point and after a third round of screening using 20 pM IgM to capture higher affinity peptides. A few phage peptide sequences had two cysteines like ϕM5 (FNVCFPFCTG, 12A1), ϕM6 (FKF CEWYGPC, 12A1), or ϕM23 (YTWYCWEEWC, 13F1), and presumably represented constrained peptides but did not provide dominant motifs (not shown in Table 1). However, the predominant feature of most of the sequenced phage was three aromatic amino acids, each separated by a nonaromatic residue. Such a motif was previously noted in phage selected by the closely related mAb 2H1 (20). The presence of this motif was used together with antibody reactivity to group the peptide sequences. Group 1 contains phage ϕM1 and ϕM2 from the hexapeptide library and ϕM8 and ϕM9 from the decapeptide library. The three aromatic positions have a mixture of tyrosines and tryptophans and a glutamate predominates at the COOH-terminus (Table 1). Phage of this group only react with mAb 12A1. Group 2 phage have only tryptophans at their aromatic positions, a constant leucine between the first two tryptophans, and a high predominance of glutamate between the last two tryptophans. Phage of this group only react with mAb 13F1. Phage ϕM4 was isolated by mAb 12A1, but only reacted by ELISA with mAb 13F1, confirming that a lower binding affinity threshold was required for selection than for the phage ELISA. The third group contains phage having three tryptophans (except ϕM30) and an extension of the constant amino acid positions at the NH_2 terminus

Table 1. *12A1 and 13F1 Selected Phage Derived from the ArXArXAr Motif*

Phage	Peptide Sequences								Selection	Absorbance		
										12A1	13F1	21D2
			Ar	**X**	**Ar**	**X**	**Ar**			*OD*		
Group 1			**Y/W**	**X**	**Y/W**	**X**	**Y/W**	**(E)**				
ϕM1			Y	D	W	L	M	F	12A1	0.70	<0.1	<0.1
ϕM2*			Y	D	W	L	Y	E	12A1	0.24	<0.1	<0.1
ϕM8		YF	W	N	Y	S	Y	E VF	12A1	>2.0	<0.1	<0.1
ϕM9		SY	W	S	S	S	W	E LY	12A1	ND	ND	ND
Group 2			**W**	**L**	**W**	**(E)**	**W**					
ϕM4		FD	W	L	W	L	W	DT	12A1	<0.1	0.9	<0.1
ϕM14		LQ	W	L	W	E	W	PRT	13F1	<0.1	1.61	<0.1
ϕM15		LD	W	L	W	E	W	AEQ	13F1	<0.1	>2.0	<0.1
ϕM16		THD	W	L	W	E	W	AS	13F1	<0.1	0.26	<0.1
ϕM17		LS	W	L	W	E	W	ELS	13F1	ND	ND	ND
ϕM18		LLDYG	W	L	W	M	W		13F1	<0.1	1.47	<0.1
Group3	**V/AV/I**		**W**	**X**	**W**	**X**	**W**					
ϕM3	S	V I	W	S	W	M	W	LD	12A1	1.51	1.50	0.76
ϕM25		A V	W	E	W	M	W	WDA	13F1/21D2	0.41	0.59	0.36
ϕM30	YK	A I	W	L	Y	M	W	I	12A1/21D2	0.20	0.27	0.14
ϕM31		A V	W	Q	W	M	W	VEY	12A1/21D2	0.68	0.79	0.72
ϕM36	RA	V I	W	A	W	M	W	E	12A1	>2.0	>2.0	0.31
ϕM26	PF	V V	W	D	W	Q	W	L	13F1/21D2	<0.1	0.12	<0.1
ϕM35	NM	V I	W	D	W	Q	W	T	12A1	0.34	0.16	0.15
ϕM19		R L	W	I	W	A	W	NVG	13F1	0.67	0.74	<0.1
	V/AV/I		**W**	**X**	**X**	**D**	**W**					
ϕM22	RV	V I	W	A	K	D	W	E	13F1	0.97	0.87	0.13
ϕM24	GLY	V I	W	S	R	D	W		3 mAbs	0.58	0.76	0.38
ϕM29	YMG	A I	W	A	G	D	W		12A1/21D2	0.25	0.39	0.24
ϕM32	LRY	V V	W	A	D	D	W		12A1/21D2	0.21	0.33	0.15

*This clone has an insert sequence identical to ϕG3 isolated by 2H1 (20).

with two aliphatic residues (except ϕM19). These phage react with both mAbs 12A1 and 13F1, often giving similar absorbance.

The relationship between mAbs 12A1 and 13F1, and mAb 21D2, another IgM which binds to the capsule in a punctate pattern (see below), was analyzed further by cross-screening; libraries that had been selected by either 12A1 or 13F1 were then submitted to two rounds of selection first with 0.2 μM and then with 2 nM of 21D2. Phage yields were 3–4% on the last round (only 0.001% for control, in absence of antibody). The sequences of these doubly selected phage fell in to group 3 (Table 1) apart from some clones with unrelated inserts (not shown). This new set of sequences refined the consensus motif of group 3 phage. The residue between the two last tryptophans is mainly a methionine, a glutamine, or an aspartate. In the latter case, the second tryptophan is no longer required.

12A1 and 13F1 Binding Pattern on C. neoformans. In previous studies, we demonstrated that IgM mAb 12A1 protected mice from lethal infection with a serotype D strain (24067; American Type Culture Collection), whereas IgM mAb 13F1 did not (5). Indirect immunofluorescence experiments revealed that mAbs 12A1 and 13F1 produced annular and punctate fluorescence patterns, respectively, suggesting that each antibody bound to a different antigenic structure in the cryptococcal capsule. To further explore this finding, we examined additional serotype D strains and several strains representing the other serotypes (Table 2 and Fig. 1). mAb binding to strains 34873 and 34874 was similar to strain 24067; the protective mAb 12A1 bound with an annular fluorescence pattern, whereas the nonprotective mAb 13F1 produced a punctate fluorescence pattern (Fig. 1, *top*). However, both 12A1 and 13F1 bound in an annular pattern to a fourth serotype D strain, J22 (Table 2). J22 is an unusual strain with an unstable karyotype (26) and a GXM structure containing a novel triad repeat (16).

Table 2. *Indirect Immunofluorescence Capsular Pattern by mAbs 12A1 and 13F1*

Strain*	Serotype	Fluorescence pattern	
		12A1	13F1
24067	D	Annular	Punctate
34873	D	Annular	Punctate
34874	D	Annular	Punctate
J22‡	D	Annular	Annular
371	A	Annular	Annular
J11	A	Annular	Annular
24064	A	Annular	Annular
SB6	A/D	Annular	Annular
24065	B	Annular	Annular
32608	C	Punctate	Punctate

*Cryptococcal strains are from the American Type Culture Collection, except J11 and J22 which are clinical isolates from New York City, SB6 which is a clinical isolate from Stony Brook, NY, and 371 which is an environmental isolate from Thailand.
‡This strain shows evidence of karyotype instability (26) and a novel GXM component observed in the nuclear magnetic resonance spectrum (16).

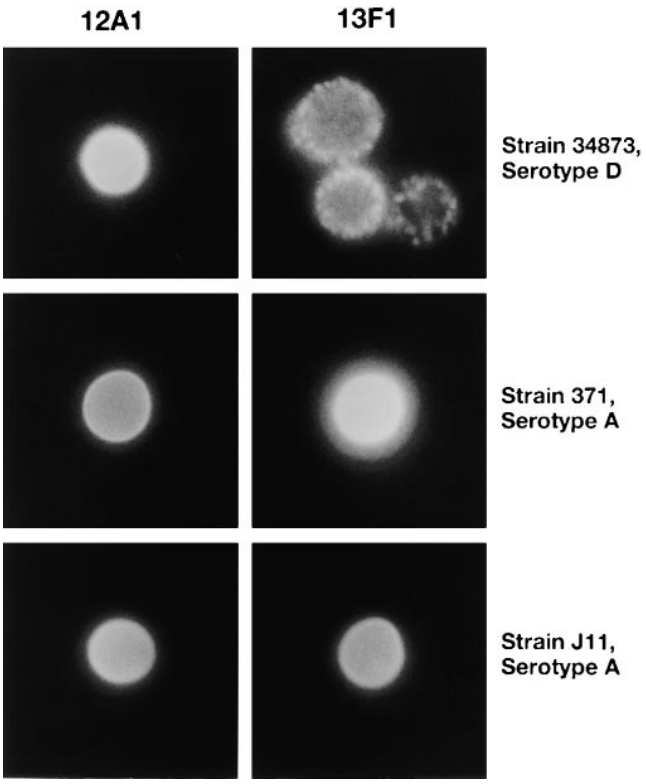

Figure 1. Indirect immunofluorescence capsular pattern with mAbs 12A1 and 13F1 on different *C. neoformans* serotype A and D strains.

In contrast to their different pattern of binding to most serotype D organisms, both mAbs 12A1 and 13F1 produced an annular fluorescence pattern on three serotype A strains and on one serotype A/D strain which contains GXM structures that are characteristic of both serotypes (Table 2 and Fig. 1). On a serotype B strain, both mAbs produced annular immunofluorescence patterns. On a serotype C strain, both mAbs produced a punctate fluorescence pattern.

To more precisely localize antibody binding, mAb binding to serotype D strain 24067 was studied by IEM (Fig. 2). Under these conditions, the capsule appeared to have structural heterogeneity with a denser outer rim linked to the cell wall by what could be described as spokes. Consistent with confocal microscopy, mAb 12A1 binding concentrated to sites at the outer rim of the polysaccharide capsule (Fig. 2 *A*). In contrast, mAb 13F1 binding was distributed throughout the capsule starting at the outer rim, penetrating deeper into the capsule along the spokes, and reaching sites almost adjacent to the fungal cell wall (Fig. 2 *B*). It is important to note that, although the antibodies were added before fixation, the capsule is fixed and dehydrated for electron microscopy, making it difficult to compare the location of the antibodies in fluorescence and electron microscopy studies.

Binding Patterns of Other Anti-GXM mAbs. To determine if other GXM-binding antibodies produced a punctate fluorescence pattern, we screened sixteen mAbs similar to 12A1 and 13F1 in their variable region sequences, and representing all isotypes except IgE. Except for the IgM mAb 21D2, all produced an annular binding pattern on both serotypes D and A, identical to the pattern produced by mAb 12A1. This included the remaining seven members of the 12A1/13F1 hybridoma genealogy that originated from a single B cell precursor, which share identical heavy and light chain CDR3 sequences and differ in a limited number of amino acids predominantly in heavy chain CDR2 (15) as well as mAbs from other fusions. IgM mAb 21D2 was isolated from a BALB/c mouse infected with a clinical isolate of *C. neoformans* (undetermined serotype) and uses a different member of the V_H 7183 family than 12A1 and 13F1, but the same V_K (18). This mAb had previously been shown to confer minimal protection against infection with serotype D strain 24067, with an average survival of 5 d versus 4 d for PBS alone (17). mAb 21D2 produces a punctate pattern on serotype D strain 24067 and an annular pattern on serotype A strain J11 (data not shown).

mAb Protective Efficacy Against C. neoformans. We first examined the ability of mAbs 12A1 and 13F1 to modify the course of infection with serotype D strain 34873 which had not been tested previously. Intraperitoneal administration of mAb 13F1 before infection decreased survival relative to controls ($P = 0.0149$), whereas mAb 12A1 prolonged survival ($P = 0.0396$ compared to controls) (Fig. 3 *A*). In addition, the animals which received mAb 13F1 died within a short time of each other (SD 0.88), in contrast to the saline controls which died over a more extended period (SD 9.53). This was similar to the previous protection experiment with serotype D strain 24067 where the standard deviations were 0.37 and 2.53 d for 13F1 and saline con-

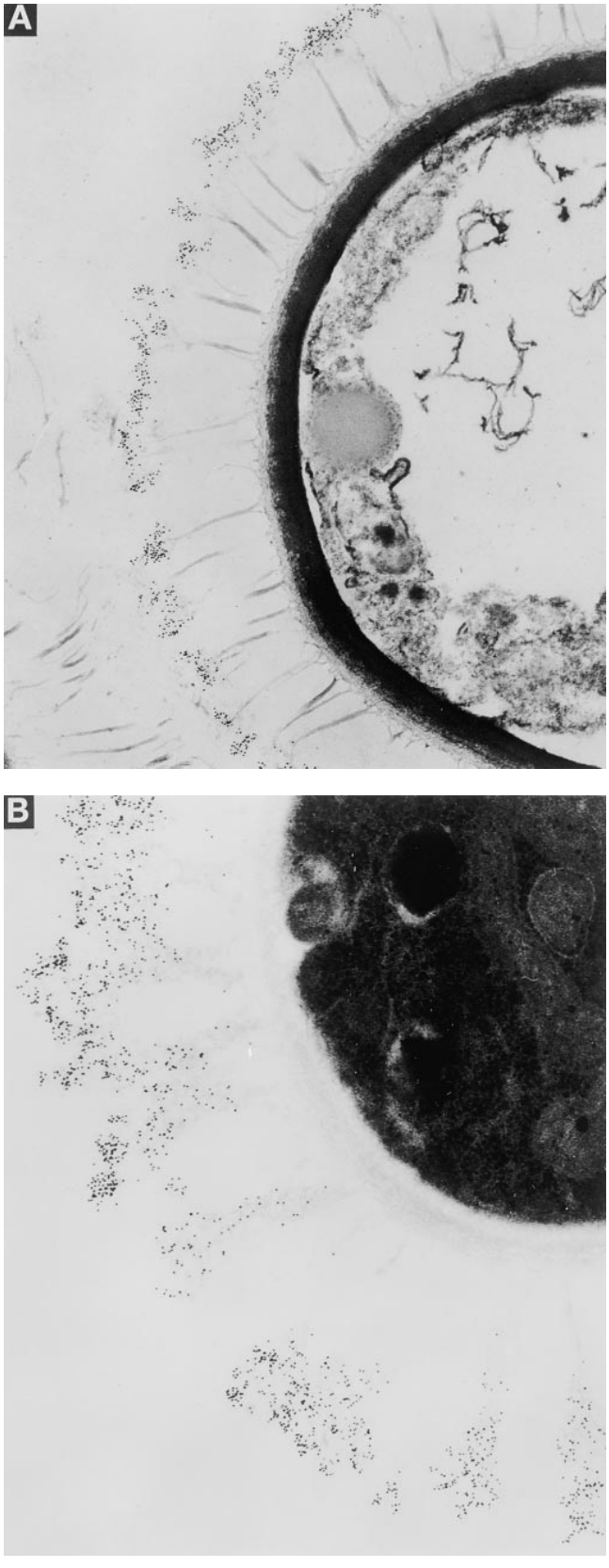

Figure 2. Immunoelectron micrograph of the cryptococcal serotype D strain 24067 capsule immunostained with gold-labeled (*A*) 12A1 and (*B*) 13F1.

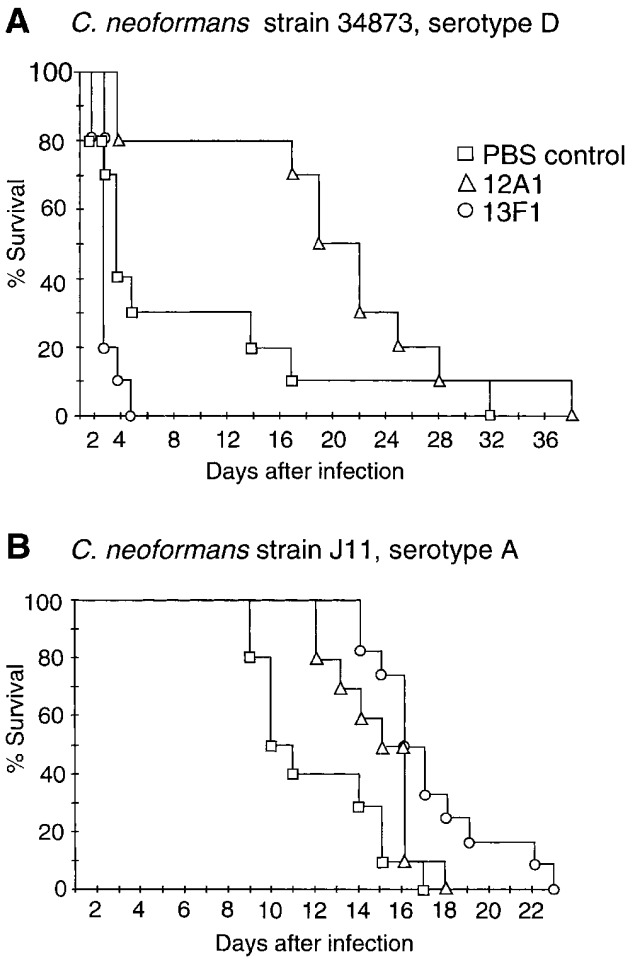

Figure 3. Survival of A/J mice after intraperitoneal treatment with the IgM mAbs 12A1 and 13F1 preceding infection with *C. neoformans.* (*A*) serotype D strain 34873, (*B*) serotype A strain J11.

trols, respectively (5). This may reflect enhanced infection in the presence of 13F1.

The fact that on serotype A, both mAbs 12A1 and 13F1 bind with the annular pattern observed for 12A1 on most serotype D organisms while 12A1 prolongs survival, raised the possibility that both mAbs would protect against serotype A infection. We tested this hypothesis using serotype A strain J11 (Fig. 3 *B*). mAb 13F1 significantly prolonged survival of mice infected with this strain compared to saline controls ($P = 0.001$). mAb 12A1 prolonged survival ($P = 0.053$), but the increase narrowly missed significance at the $P = 0.05$ level. Nevertheless, mAb 13F1 clearly differs in its ability to confer protection against the strains representing the A and D serotypes that we have tested since it prolongs survival of animals infected with the J11 A strain and enhances infection with the 34873 D strain.

Phagocytosis Assays. mAbs 12A1 and 13F1 were tested for their ability to promote opsonization of organisms by macrophages. mAb 12A1 significantly enhanced phagocytosis when added to monolayers of J774.16 and strain 24067 cells (Fig. 4 *A*). Using the same conditions, 13F1 did not enhance phagocytosis, even at concentrations as high as 25

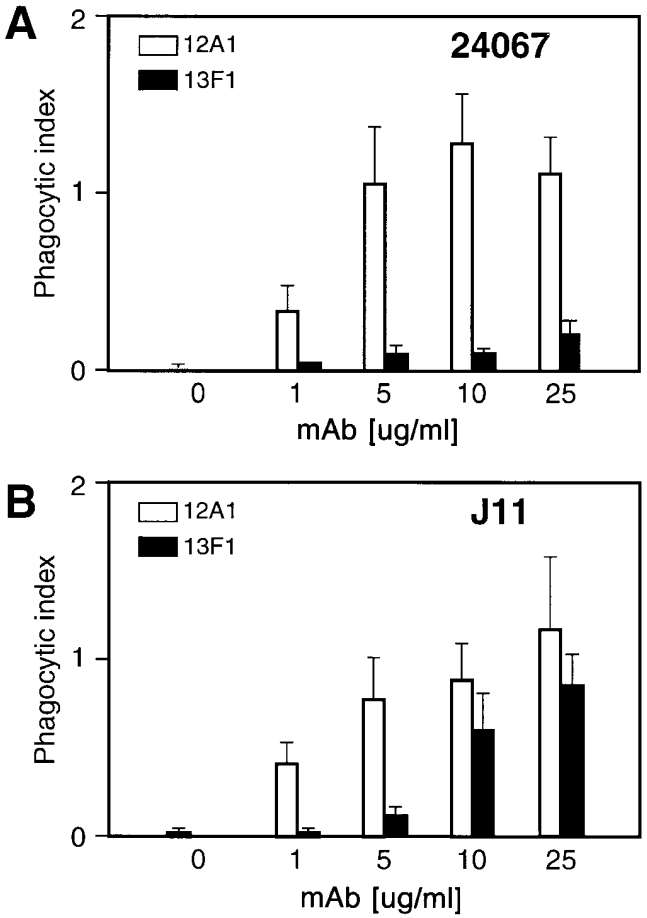

Figure 4. Opsonic activity of mAbs 12A1 and 13F1 in the presence of J774.16 macrophages on (*A*) *C. neoformans* serotype D strain 24067, and (*B*) *C. neoformans* serotype A strain J11.

μg/ml. With the J11 serotype A organisms and low antibody concentrations, 12A1 was still more effective than 13F1. However, above 10 μg/ml, both mAbs promoted significant phagocytosis (Fig. 4 *B*). Annular binding is therefore associated with more effective opsonization than punctate binding.

Discussion

We recently showed that mAbs 12A1 and 13F1, two IgM anti-GXM antibodies with the same apparent avidity, were quite different in their ability to prolong the life of mice infected with strain 24067 serotype D *C. neoformans* (5). These differences in protection were attributed to differences in epitope specificity and were associated with different patterns of capsular binding. Cryptococcal polysaccharide is composed of a linear (1→3)-α-d-mannopyranan substituted with β-d-glucopyranosyluronic acid and varying degrees of β-d-xylopyranosyl and 6-*O*-acetyl groups, depending on the serotype. The order of reactivity with GXM from the four serotypes for mAb 12A1 is A>B>D>C, and for mAb 13F1 it is A>B>C>D (27). The relative degree of xylose substitution among the *C. neoformans* serotypes is C>B>A>D, and the degree of *O*-acetylation is A>B>D>C (28). Thus, it is not possible to identify the mAb epitope on the basis of reactivity with GXM of the different serotypes. Epitope analysis is further complicated by the difficulty in generating oligosaccharide subunits of GXM capable of inhibiting mAb binding.

To compare the fine specificities of these anti-GXM antibodies and gain further insight into the antibody binding site, we have used phage display peptide libraries to obtain peptide mimetics of GXM epitopes. For example, the protective IgG1 2H1 bound to several peptide motifs (20). Two of these motifs (2H1 motif 1, (E)TPXWM/LM/L; and 2H1 motif 2, W/YXWM/LYE) cross-reacted with many anti-GXM antibodies, but not with 13F1. The three-dimensional structure of 2H1 bound to a peptide of 2H1 motif 1 showed the antibody binding site to be a hydrophobic pocket that was largely, but not completely, occupied by the peptide (Young, A.C.M., P. Valadon, A. Casadevall, M.D. Scharff, and J.C. Sacchetini, manuscript in preparation). Most sequences identified by both mAbs 12A1 and 13F1 contain three aromatic amino acids, each separated by one residue. This pattern (ArXArXAr) was also present in peptides selected by 2H1 (20) indicating that mAbs 12A1 and 13F1 bound peptides at the same location in the antibody binding pocket and probably in a similar way. More precisely, just as with the 2H1 motifs, there is probably a type II β turn in the backbone of the bound form of the peptide since such a structure is required to fit the anti-GXM antibody binding pocket (Young, A.C.M., P. Valadon, A. Casadevall, M.D. Scharff, and J.C. Sacchetini, manuscript in preparation). As a result, the peptide residues group in a compact structure with all lateral chains pointing down toward the binding pocket where they are in close contact with the antibody residues.

The peptides selected by 12A1 and 13F1 from two different phage libraries could be grouped into three sets according to their sequence and their reactivity with the mAbs. Group 1 peptides bind only 12A1 and share the motif W/YXW/YXW/YE. This motif is reminiscent of the 2H1 motif W/YXWM/LYE (20) and contains ϕM2, a phage bearing a peptide sequence identical to ϕG3, a phage that had been isolated previously by mAb 2H1. Phage ϕG3 was already noted to have the largest cross-reactivity among anti-GXM antibodies. In fact, cross-screening of the decapeptide library with several anti-GXM antibodies shows that the 2H1 motif W/YXWM/LYE can define the epitope bound by most anti-GXM mAbs elicited by GXM-TT (Valadon, P., and M.D. Scharff, manuscript in preparation). mAb 12A1 reacts with peptides of this motif, gives the same fluorescent pattern as 2H1 on serotypes A and D, and has a similar protective effect. This suggests that 12A1 and 2H1 bind to the same protective epitope which is located on the outer part of the cryptococcal capsule. On the other hand, mAb 13F1 did not cross-react with any of the 2H1-isolated phage (20), but isolated group 2 phage bearing a specific sequence motif (WLW[E]W) that is distinguished from group 1 by the leucine between the first two aromatic residues and the absence of tyrosine. This result suggests the presence of a significant structural difference in 13F1 bind-

ing site that makes the specificity of 13F1 distinct from 12A1 or related antibodies like 2H1.

When examined by indirect immunofluorescence using confocal microscopy, mAb 12A1 bound to the outer rim of the polysaccharide capsule giving an annular pattern of binding, while mAb 13F1 bound in discrete areas throughout the capsule with a punctate pattern of binding. IEM revealed a structural heterogeneity in the polysaccharide capsule of *C. neoformans* with an outer rim connected to the cell wall by spoke-like structures. According to the indirect immunofluorescence observation, mAb 12A1 bound to the outer rim part of the capsule. On the other hand, 13F1 binding occurred at the outer rim but was also distributed throughout the capsule and almost reached sites alongside the cell wall (Fig. 2). Since all of these results were obtained with polymeric IgM antibodies in indirect assays using anti-IgM antisera, it is not clear whether these structures exist as such, are the result of flexible polysaccharide molecules bearing distinct epitopes that may be present in different densities, or are located in different parts of the capsule and are aggregated by decavalent IgM antibodies. We have not identified IgG antibodies that bind with high enough affinity to the 13F1 epitope to allow us to generate Fab′ molecules that could be used in direct immunofluorescence to resolve this issue.

The punctate pattern of binding with mAb 13F1 was observed on other serotype D organisms, except for the J22 strain which has an unusual polysaccharide structure consisting of a novel triad repeat (16). In contrast, mAbs 12A1 and 13F1 both produced annular fluorescence patterns when bound to serotype A strains. The change in mAb 13F1 binding pattern from punctate to annular correlated with a shift from nonprotective or even enhancing on serotype D, to protective on serotype A. This led us to the hypothesis that annular binding was associated with enhanced phagocytosis and protection, and suggested that the binding of the IgM molecule to the surface of the organism was critical for Fc-mediated effector functions.

The ability of mAbs 12A1 and 13F1 to opsonize *C. neoformans* was studied on J774.16 cells, a well-established macrophage-like cell line with antibody-mediated anticryptococcal activity (25). mAb 12A1 was more effective than mAb 13F1 in promoting phagocytosis of serotype A and D organisms, and annular binding was associated with an increase in the ability of mAb 13F1 to opsonize *C. neoformans* for phagocytosis. The mechanism by which IgM opsonize *C. neoformans* in the absence of complement is not understood, but could be due to an unrecognized IgM receptor on J774.16 cells, mAb-induced structural changes in the polysaccharide capsule that reduce its inherent antiphagocytic properties, or complement-mediated phagocytosis using complement synthesized by the macrophage-like cell (29). Whatever the mechanism, the phagocytosis results indicate that annular and punctate binding to the polysaccharide capsule are associated with differences in opsonization. In vivo, IgM mAbs like 12A1 may enhance clearance of fungal cells by fixing complement component C3b which serves as a potent opsonin for *C. neoformans* (30). mAb 13F1 appears to be both near the surface and sequestered within the capsule at locations where it may interact with complement in a different manner (31). These results are similar to the finding of Jones and Fischetti (2) that the location of mAb binding within the M6 surface protein of group A streptococci determined whether or not an mAb effectively opsonized bacteria. It has also been shown that epitope density may influence the ability of the Fc region to activate complement (32). Despite seemingly similar binding densities of 12A1 and 13F1 on the immunoelectron micrographs, it is important to note that IEM is an indirect assay and the actual epitope densities of mAbs 12A1 and 13F1 could be quite different.

When we examined 16 additional anticryptococcal mAbs with closely related V region sequences for their binding pattern to polysaccharide capsule, only the IgM mAb 21D2, which is also not protective against serotype D infection, produced a binding pattern on serotypes D and A similar to that of mAb 13F1. While mAb 13F1 was isolated in response to immunization with GXM-TT, mAb 21D2 arose in response to infection, demonstrating that nonprotective antibodies can arise naturally as well as in response to immunization (18). Based on fluorescence pattern alone, it is impossible to determine whether mAbs 13F1 and 21D2 bind the same epitope. We tried to link 21D2 to 13F1 or 12A1 by sequential selection of peptide on phage using the mAbs. This resulted in the isolation of group 3 peptides that reacted identically with the three mAbs (Table 1), but did not show any cross-reactivity with a large panel of anti-GXM antibodies (data not shown). However, de-*O*-acetylation of serotype D GXM causes a loss of binding for mAb 13F1, but not for 21D2, suggesting that they have different fine specificities and could even bind distinct epitopes (33). mAbs 13F1 and 21D2 use the same V_κ5.1 gene but distinct members of the V_H7183 gene family. Among 29 BALB/c mAbs recovered from three separate fusions, mAbs 13F1 and 21D2 share a common somatic mutation at position 33 in the heavy chain converting a phenylalanine in the consensus sequence to a tyrosine. This shared tyrosine may be responsible for a shift in interaction with cryptococcal polysaccharide.

Cherniak et al. demonstrated structural variations in the capsular GXM from initial and relapse isolates recovered from individual patients (16). In particular, they identified a patient with an initial isolate that was serotype A/D, serotype A at the time of the first relapse, and then serotype D at the time of the second relapse. This observation suggested that variations in polysaccharide structure among sequential isolates from one patient may reflect selective influences of the host immune system. Our observation provides a mechanism by which changes in serotype could happen in vivo. Protective antibodies could select antigenic variants of *C. neoformans* which neutralize antibody by sequestration at sites where it is unable to carry out effective protective functions. This phenomenon, if it occurs during infection, has important implications for the design of vaccines and the use of passively administered mAbs in cryptococcal infection. The results presented here emphasize the importance of evaluating the protective potential of antibodies on multiple representatives of different strains.

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Address correspondence to Dr. Matthew D. Scharff, Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

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